Epidermal Growth Factor Receptor Regulates Aberrant Expression of Insulin-Like Growth Factor-Binding Protein 3

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ABSTRACT

Epidermal growth factor receptor (EGFR) is frequently overexpressed in esophageal carcinoma and its precursor lesions. To gain insights into how EGFR overexpression affects cellular functions in primary human esophageal cells, we performed gene expression profiling and identified insulin-like growth factor-binding protein (IGFBP)-3 as the most up-regulated gene. IGFBP-3 regulates cell proliferation through both insulin-like growth factor-dependent and independent mechanisms. We found that IGFBP-3 mRNA and protein expression was increased in EGFR-overexpressing primary and immortalized human esophageal cells. IGFBP-3 was also up-regulated in EGFR-overexpressing cells in organotypic culture and in EGFR transgenic mice. Furthermore, IGFBP-3 mRNA was overexpressed in 80% of primary esophageal squamous cell carcinomas and 60% of primary esophageal adenocarcinomas. Concurrent up-regulation of EGFR and IGFBP-3 was observed in 60% of primary esophageal squamous cell carcinomas. Immunohistochemistry revealed cytoplasmic localization of IGFBP-3 in the preponderance of preneoplastic and neoplastic esophageal lesions. IGFBP-3 was also overexpressed in esophageal cancer cell lines at both mRNA (60%) and protein (40%) levels. IGFBP-3 secreted by cancer cells was capable of binding to insulin-like growth factor I. Functionally, epidermal growth factor appeared to regulate IGFBP-3 expression in esophageal cancer cell lines. Finally, suppression of IGFBP-3 by small interfering RNA augmented cell proliferation, suggesting that IGFBP-3 may inhibit tumor cell proliferation as a negative feedback mechanism. In aggregate, we have identified for the first time that IGFBP-3 is an aberrantly regulated gene through the EGFR signaling pathway and it may modulate EGFR effects during carcinogenesis.

INTRODUCTION

Among cancers, esophageal carcinoma is one of the leading causes of cancer-related mortality worldwide. Esophageal malignancies are predominantly of two types: squamous cell carcinoma and adenocarcinoma. Etiologically, the former is associated with cigarette smoking and alcohol, whereas the latter is associated with Barrett’s esophagus, which is characterized by intestinal metaplasia of the esophageal squamous epithelium caused in part by acid reflux. Common genetic lesions identified in both forms of esophageal cancer include epidermal growth factor receptor (EGFR) and cyclin D1 overexpression, inactivation of p53 and p16INK4a, and telomerase activation (1).

EGFR is a receptor tyrosine kinase whose biochemical and structural properties have been extensively studied (2). EGFR critically regulates a number of cellular functions, including cell proliferation, survival, differentiation, migration, cell–cell adhesion, and cell–extracellular matrix interactions. EGFR is a classical proto-oncogene that can transform NIH3T3 fibroblasts in a ligand-dependent fashion (3). EGFR overexpression, partially accounted for by gene amplification, is found in up to 80% of esophageal squamous cell carcinomas (ESCCs) and esophageal adenocarcinomas (EACs), as well as their precursor lesions, such as squamous dysplasia and Barrett’s esophagus, respectively (4, 5). However, most studies on EGFR biological functions have been carried out in rodent fibroblasts or human cancer cell lines, thus hindering insights into the molecular and physiologic mechanisms through which EGFR overexpression contributes to immortalization and malignant transformation.

We have recently isolated and characterized primary human esophageal cells (6). Moreover, using retrovirus-mediated transduction, we established stable cell lines expressing EGFR and/or the catalytic subunit of human telomerase (hTERT; refs. 6, 7). Primary esophageal cells overexpressing EGFR demonstrated unique cell biological properties, including increased migration associated with induction of matrix metalloproteinase-1, pronounced cell aggregation through enhanced functions of E-cadherin, and basal cell hyperplasia in organotypic culture (6). However, EGFR alone failed to immortalize primary esophageal cells. By contrast, hTERT-mediated telomerase activation resulted in immortalization of primary human esophageal cells without affecting the p53 and pRb pathways (7).

Insulin-like growth factor-binding protein (IGFBP)-3, one of six IGFBPs, is the major carrier protein for insulin-like growth factor (IGF-I) or IGF-II in circulation. IGFBP-3 protein has molecular masses of 43 to 45 kDa, depending on posttranslational modifications such as glycosylation and phosphorylation. It exists as a component of a 150-kDa ternary complex comprising an 85-kDa acid labile glycoprotein subunit and IGF-I or IGF-II (8, 9). Various cell types including fibroblasts, endothelial cells, and epithelial cells secrete IGFBP-3 (10–12). Although many endogenous and pharmacological agents, including peptide growth factors, cytokines, and hormones, have been shown to induce IGFBP-3, how IGFBP-3 expression is regulated remains largely undetermined.

IGFBP-3 has been demonstrated to regulate cell proliferation through both IGF-dependent and independent mechanisms (9, 13). IGFBP-3 is known to be up-regulated in senescent cells or mitotically quiescent cells (11, 12, 14). However, little knowledge is available regarding IGFBP-3 expression and function in cancer. Depending on the experimental context, IGFBP-3 has been shown to possess growth-stimulatory, antiproliferative, or proapoptotic activities in vitro. In transgenic mice, ubiquitous expression of IGFBP-3 resulted in growth retardation, suggesting that IGFBP-3 may impair the growth-promoting functions of IGFs in vivo (15). However, tissue-specific targeted expression of the IGFBP-3 transgene by metallothio-
nein-1 induced selective organomegaly of the spleen, liver, and heart (16). In other IGFBP-3 transgenic mice, the mammary gland underwent delayed involution after lactation, suggesting that IGFBP-3 overexpression potentiated the antiapoptotic activities mediated by IGFs (17).

To gain further insights into how EGFR overexpression affects cellular functions of primary human esophageal cells, we carried out gene expression profiling and identified IGFBP-3 as a critically upregulated gene. We find that IGFBP-3 is frequently overexpressed in esophageal cancer cell lines and primary esophageal tumors. We also demonstrate a basis for IGFBP-3 regulation by EGFR. Moreover, IGFBP-3 appears to negatively regulate cell proliferation in esophageal cancer cells. Indeed, the novel findings described herein may help to explain how EGFR through IGFBP-3 may modulate cell growth but not induce immortalization and malignant transformation.

MATERIALS AND METHODS

Tissue Samples. Esophageal tissue samples were procured via surgery from the Okayama University Hospital (21 cases) and the Hospital of the University of Pennsylvania through the Cooperative Human Tissue Network (11 cases). Eight and 24 cases were pathologically diagnosed as EAC and ESCC, respectively. All of the ESCC tissues contained lesions consistent with squamous dysplasia. Paraffin blocks representing tumors and adjacent normal tissues were available from 6 of 8 EAC cases and 17 of 24 ESCC cases for immunohistochemistry. Paired frozen tissues representing tumors and adjacent normal tissues were available from 7 of 8 EAC cases and 19 of 24 ESCC cases for RNA or protein analyses. All of the clinical materials were obtained from patients who provided informed consent in accordance with institutional review board standards and guidelines.

EGFR Transgenic Mice. Generation of EGFR transgenic mice was described previously (6). The EGFR transgene was targeted in the oral-esophageal epithelium in a tissue-specific fashion with the Epstein-Barr virus ED-L2 promoter (18). The EGFR transgenic mice and age-matched wild-type mice were sacrificed at age 6 months for immunohistochemistry. All transgenic mouse experiments were carried out in accordance with the standards and guidelines of the Institutional Animal Care and Use Committee at the University of Pennsylvania.

Cell Lines. Fifteen human esophageal carcinoma cell lines (TE series, T.T., HCE4, and HCE7) and six nonsophageal cell lines (Hep2G, PANC-1, MCF7, HeLa, A431, and HaCaT) were cultured under standard conditions as described previously (19). TE7 cells were derived from adenocarcinoma, whereas the other esophageal carcinoma cell lines were established from ESCCs. EPC1 and EPC2 are primary human esophageal keratinocytes that have been described previously (6, 7). EPC1 and EPC2 cells were grown at 37°C under 5% CO2 in serum-free medium (Keratinocyte-SFM) supplemented with 40 μg/mL bovine pituitary extract and 1 ng/mL epidermal growth factor (EGF; Invitrogen, Carlsbad, CA). Esophageal epithelial cells (designated mouse esophageal keratinocytes) were isolated from the EGFR transgenic mouse and the control mouse and grown in culture as described previously (20). Cells were counted using Coulter Z1 counter (Beckman Coulter Inc., Fullerton, CA). Cell viability was determined by trypsin blue exclusion. Conditioned medium (CM) was harvested by incubating subconfluent cells for an indicated time period with full Keratinocyte-SFM or Dulbecco’s modified Eagle’s medium (DMEM) containing either 0% fetal calf serum (FCS) or 0.5% FCS. To concentrate CM, Centricon YM-10 centrifugal filter units (Amicon, Beverly, MA) were used to isolate total RNA from cultured cells and snap-frozen tissues, respectively. RNA was treated with DNase I (Invitrogen). The integrity of total RNA was determined by 1% formaldehyde–agarose gel electrophoresis. cDNA synthesis was carried out with the Superscript First Strand Synthesis System (Invitrogen) using 3.3 μg (cell RNA) or 5 μg (tissue RNA) of total RNA as a template. The cDNA synthesis reactions without reverse transcriptase yielded no amplinons in the polymerase chain reaction (PCR) reactions described below.

Gene Expression Profiling. Gene expression profiling of EPC2-EGFR and EPC2-GFP cells was carried out using the Affymetrix HG-U95A Human GeneChip array (Affymetrix, Santa Clara, CA). Preparation of cRNA, hybridization, and scanning of the arrays were performed according to manufacturer’s protocols. Briefly, 5 μg of total RNA were primed by an oligo(DT)25 primer containing a T7 RNA polymerase promoter 3′ to the poly(T) (Geneset, La Jolla, CA) and converted to first-strand cDNA using Superscript II reverse transcriptase (Invitrogen). Second-strand cDNA synthesis was followed by in vitro transcription for linear amplification of each transcript incorporating biotin-11-UTP and biotin-16-UTP (Enzo, Farmington, NY) to generate biotin-labeled cRNA. The labeled cRNA was purified over RNeasy columns. Fifteen micrograms of cRNA (A were fragmented to ≤200 nucleotides at 90°C for 35 minutes in 40 mmol/L Tris-acetate (pH 8.1), 100 mmol/L, potassium acetate, and 30 mmol/L magnesium acetate; heat-denatured at 99°C for 5 minutes; and hybridized for 16 hours at 45°C to the HG-U95A microarray in 200 μL of hybridization mixture consisting of 100 mmol/L 4-morpholinoethanesulfonic acid, 1 mol/L NaCl, 20 mmol/L EDTA, 0.01% Tween 20, 0.1 mg/mL herring sperm DNA (Promega, Madison, WI), and 500 μg/mL acetylated bovine serum albumin (Invitrogen). The microarray was then washed at low (0.9 mol/L NaCl, 60 mmol/L /NaHPO4, 6 mmol/L EDTA, and 0.01% Tween 20) and high (100 mmol/L 4-morpholinoethanesulfonic acid, 0.1 mol/L NaCl, and 0.01% Tween 20) stringency and stained with streptavidin–phycoerythrin (Molecular Probes, Eugene, OR). Fluorescence was amplified by adding biotinylated antistreptavidin and an additional aliquot of streptavidin–phycoerythrin stain. The GeneArray scanner (Affymetrix) was used to collect fluorescence signal at 3 μm resolution after excitation at 570 nm. The average signal from two sequential scans was calculated for each microarray feature. The Affymetrix array data were analyzed using Affymetrix Microarray Suite 5.0 (Affymetrix) and Genespring 5.0 (Silicon Genetics, Redwood City, CA), programs designed for the analysis of high-throughput microarray expression data. Genes that are absent in all samples were removed from consideration. The list of genes was condensed to genes that changed ≥3-fold (whether up or down) between EPC2-GFP and EPC2-EGFR cells.
Real-Time Reverse Transcription-Polymerase Chain Reaction. Real-time PCR was performed using SYBR green reagent (PE Applied Biosystems, Foster City, CA) and the ABI PRISM 7000 Sequence Detection System (PE Applied Biosystems) according to the manufacturer’s instructions. The following oligonucleotides were used as primers: (a) human IGFBP-3, 5’-CCAT-GACTGAGGAAAGGACCT-3’ (BP3-2218-F; exon 4; nucleotides 10487–10508; forward primer) and 5’-TCGACAGGGCCAGTCTC-3’ (BP3-2325-R; exon 4; nucleotides 10552–10594; Genbank accession no. M53878; reverse primer); (b) mouse IGFBP-3, 5’-GAGTTGGAAGACGGACGTGAGTCTC-3’ (mouse5252-F; forward primer) and 5’-GATAAGGGAGTGCAGAACATTGTTG-3’ (mouse5393-R; Genbank accession no. NM_008343; reverse primer); (c) human β-actin, 5’-CCCTGCAACCCAGCAATCT-3’ (exon 5; hBAC-F; forward primer) and 5’-GCCGATACCCAGGAATGACT-3’ (exon 6; hBAC-R; reverse primer); (d) human glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 5’-GTTGTTCTCTGCTGATACAA-3’ (exon 7; hGAPDH-F; forward primer) and 5’-GTTGCTGAACCTATTTGTT-3’ (exon 8; hGAPDH-R; reverse primer); and (e) mouse GAPDH, 5’-GTTGTTCTCTGAGCCACTA-3’ (mGAPDH-F; forward primer) and 5’-CCAGGGAATGCTGTCAGAAGTT-3’ (mGAPDH-R; reverse primer). β-Actin was used as an internal control for human tissues. GAPDH was used as an internal control for cultured cells as well as mouse tissues. PCR conditions were optimized by performing primer matrix reactions and generating standard curves for IGFBP-3, β-actin, and GAPDH. All PCR reactions were performed in triplicate in a 25-μL total volume using 500 nmol/L each of forward and reverse primers. One microliter of cDNA was used as template per reaction to detect human and mouse IGFBP-3 mRNA, whereas 0.125 μL of cDNA was used for β-actin and GAPDH. The relative expression level of IGFBP-3 mRNA between normal and tumor was calculated by normalizing to the normal CT, where \( \Delta CT \) represents the difference between the average IGFBP-3 Ct value and the average β-actin Ct value within a given tissue. \( \Delta CT \) represents the difference between the \( \Delta CT \) values for normal and tumor. The normalized IGFBP-3 expression level for normal tissues was set to 1. Likewise, the relative expression level of IGFBP-3 mRNA among cell lines was determined by normalizing to the GAPDH mRNA level. EPC2 [3 population doublings (PDs)] was used as a normal control. Thus, the normalized IGFBP-3 mRNA expression level in EPC2 (3 PDs) was set to 1. Data were analyzed using ABI PRISM 7000 sequence detection system software (PE Applied Biosystems).

Antibodies. Affinity-purified goat antihuman IGFBP-3 (DSL-R00536; Diagnostic Systems Laboratories, Inc., Webster, TX), affinity-purified rat anti-mouse IGFBP-3 (138202; R&D Systems Inc., Minneapolis, MN), affinity-purified goat antihuman IGFBP-4 (DSL-R00637; Diagnostic Systems Laboratories, Inc.), anti-EGFR Ab-12 mouse monoclonal antibody (Cocktail R194/84; NeoMarkers, Union City, CA), anti-β-actin (Sigma), anti-tubulin (DakoCytomation, Carpinteria, CA), and anti-DNA polymerase and endonuclease antibodies were used as capture antibodies, 

The reaction was terminated by 50 μL/well of 2N H2SO4, and the absorbance of each well was immediately determined using the \( V_{\text{max}} \) kinetic microplate reader (Molecular Devices Corp., Sunnyvale, CA) at 450 nm with correction at 546 nm.

Immunohistochemistry. Immunohistochemistry for IGFBP-3 was performed with the Vectastain Elite kit (Vector Laboratories, Burlingame, CA) following the manufacturer’s protocol. In brief, paraffin sections were deparaffinized with xylene, hydrated in descending ethanol solutions, and then placed in a microwave in 10 mmol/L citric acid buffer endogenous peroxidase was quenched using hydrogen peroxide before sections were blocked in avidin B reagent and biotin blocking reagent. Sections were incubated with primary antihuman IGFBP-3 mouse monoclonal antibody (clone 48728111; R&D Systems Inc.) at a 1:250 dilution and with biotinylated anti-Goat IG 50 μL/well streptavidin-horseradish peroxidase, and 100 μL/well of a 1:1 mixture of H2O2 and tetramethylbenzidine. The reaction was terminated by 50 μL/well of 2N H2SO4, and the absorbance of each well was immediately determined using the \( V_{\text{max}} \) kinetic microplate reader (Molecular Devices Corp., Sunnyvale, CA) at 450 nm with correction at 546 nm.

Enzyme-Linked Immunosorbent Assay. Enzyme-linked immunosorbent assay (ELISA) was performed using human IGFBP-3 DuoSet ELISA development systems (R&D Systems Inc., Minneapolis, MN), according to the manufacturer’s instructions. Briefly, a BD Falcon 96-well microplate (BD Biosciences, San Jose, CA) was coated with mouse antihuman IGFBP-3 capture antibody and blocked with 5% Tween 20 and 5% sucrose in PBS containing 0.05% NaN3 at room temperature for 16 hours. After washing with wash buffer consisting of 0.05% Tween 20 and 5% sucrose in PBS [137 mmol/L NaCl, 2.7 mmol/L KCl, 8.1 mmol/L Na2HPO4, and 1.5 mmol/L KH2PO4 (pH 7.4)] containing 0.1% bovine serum albumin and 10 μL/well biotinylated goat antihuman IGFBP-3 antibody, 100 μL/well streptavidin-horseradish peroxidase, and 100 μL/well of a 1:1 mixture of H2O2 and tetramethylbenzidine. The reaction was terminated by 50 μL/well of 2N H2SO4, and the absorbance of each well was immediately determined using the \( V_{\text{max}} \) kinetic microplate reader (Molecular Devices Corp., Sunnyvale, CA) at 450 nm with correction at 546 nm.

Western Blotting. Western blotting was carried out as described previously (6). In brief, cells were homogenized with a radioimmunoprecipitation assay buffer containing 10 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 1% Nonidet P-40, 0.1% SDS, 0.1% sodium deoxycholate, 1 mmol/L EDTA, 2 mmol/L Na2VO4, 1 mmol/L phenylmethylsulfonyl fluoride, and a protease inhibitor mixture tablet (Complete; Roche Applied Science, Indianapolis, IN) and cleared by centrifugation at 14,000 rpm at 4°C for 15 minutes. The total protein sample (20 μg) was denatured and fractionated on a NuPAGE Bis-Tris 4% to 12% gel with NuPAGE MOPS running buffer using the NuPAGE system (Invitrogen) and electrotransferred to polyvinylidene difluoride membrane (Immobilon-P). The membrane was then fixed with 3% TBS containing 3% Nonidet P-40 for 30 minutes at 4°C and then rinsed once with TBS containing 1% bovine serum albumin for 2 hours at 4°C. The membrane was incubated with 1 μL of 3-[3H]thymidine, 7713 [3H]Thymidine Incorporation Assay. To assess cell proliferation, DNA synthesis was measured by incubating cells with 1 μCi of [methyl-3H]thymidine (1 μCi/mL/well; Perkin-Elmer Life Sciences Inc., Boston, MA) for 4 hours in 12-well tissue culture dishes. Cells were washed three times with cold PBS, washed once with cold 10% trichloroacetic acid, and washed three times with 5% trichloroacetic acid. Cells were lysed with 0.5% NaOH on ice for 10 minutes and neutralized with 0.5% HCl. The cell lysate was supplemented with 0.1% trichloroacetic acid, incubated on ice for 20 minutes, and filtered with glass microfiber filters (Whatman, Kent, United Kingdom) using a sampling vacuum manifold (Millipore, Billerica, MA). The filters were rinsed three
times with ethanol, dried at 80°C for 45 minutes, and suspended in 10 mL of Ready Value Liquid Scintillation Cocktail (Beckman Coulter Inc.) to measure radioactivity with a LS 6500 Multi-Purpose Scintillation Counter (Beckman Coulter Inc.).

Statistical Analyses. Simple linear regression analysis was performed to compare EGFR level and IGFBP-3 level between tumors and adjacent normal tissues. Student’s t test was used to compare data between two groups. Data represent the mean ± SE. Ps of <0.05 were considered statistically significant.

RESULTS

EGFR Induces IGFBP-3 in Primary and hTERT-Immortalized Human Esophageal Cells. To identify genes expressed differentially in EPC2-EGFR cells compared with control EPC2-GFP cells, we used an Affymetrix HG-U95A Human GeneChip consisting of ~12,500 probe sets. Two independent data analysis programs, Affymetrix Microarray Suite 5.0 and Genespring 5.0, extracted 23 and 35 genes, respectively, as ~3-fold up-regulated in EPC2-EGFR cells. IGFBP-3 was the most highly up-regulated gene in EPC2-EGFR cells.

To verify the gene array result for IGFBP-3 in EPC2-EGFR cells, real-time reverse transcription (RT)-PCR and Western blotting were carried out. EPC2-EGFR cells expressed 4.4-fold more IGFBP-3 mRNA than control cells (Fig. 1A). This was corroborated by Northern blotting (data not shown). Western blotting also revealed a 3.5-fold induction of IGFBP-3 protein in EPC2-EGFR cells compared with control cells (Fig. 1B). To further confirm IGFBP-3 up-regulation in EGFR-overexpressing cells, retrovirus transduction was carried out in an independent fashion. In EPC2, EPC2-hTERT, and another primary human esophageal line, EPC1, EGFR transduction resulted in IGFBP-3 mRNA and protein induction (Fig. 1A and B). We also confirmed by Western blotting that the IGFBP-3 full-length form is secreted in CM from EPC2, EPC2-hTERT, and EPC1 cells transduced with EGFR (Fig. 1C). An ELISA confirmed that the CM from these EGFR-overexpressing cells contained 2.5- to 5-fold higher levels of IGFBP-3 than media from control cells (Fig. 1D). Furthermore, immunocytochemistry revealed that both EGFR and IGFBP-3 are more intensely expressed in the EGFR-transduced cells than control cells (Fig. 1E and F; data not shown). It should be noted that there was no significant variation in IGFBP-3 expression between cells, even though the primary cell derivatives were pooled after retrovirus infection.
IGFBP-3 Is Up-Regulated in EGFR-Overexpressing Reconstituted Esophageal Epithelium and the Esophagus of Transgenic Mice. To determine the role of EGFR in the induction of IGFBP-3 in a tissue context, we examined IGFBP-3 expression in organotypic culture (also referred to as reconstruct) and ED-L2 EGFR transgenic mice. We have recently shown that EGFR overexpression results in esophageal epithelial hyperplasia in these model systems. In organotypic culture, EPC2-EGFR cells formed a taller epithelium than control cells with increased basal cell proliferation, which was antagonized by AG1478, an EGFR-specific tyrosine kinase inhibitor (6). Immunohistochemistry revealed an extended stratification of IGFBP-3–positive cells in the EPC2-EGFR epithelium (Fig. 2A), whereas IGFBP-3 expression was restricted to the basal compartment of the control cell reconstruct (Fig. 2B).

In the ED-L2 EGFR transgenic mice, real-time RT-PCR on RNA samples from mouse esophagus also demonstrated a marked induction of IGFBP-3 mRNA compared with wild-type littermates (Fig. 2C). When esophageal epithelial cells were isolated from the ED-L2 EGFR transgenic mice, they maintained EGFR overexpression and IGFBP-3 induction (Fig. 2D and E). These findings suggest that EGFR induces IGFBP-3 in esophageal epithelial cells both in vitro and in vivo.

We also observed that IGFBP-3 mRNA was initially down-regulated when presenescent EPC2 cells (42 PDs) were transduced with hTERT. However, IGFBP-3 expression was increased in immortalized EPC2-hTERT cells at a later PD (>200 PDs; data not shown). This prompted us to investigate whether or not IGFBP-3 is up-regulated in esophageal cancer cells that are typically associated with EGFR overexpression and telomerase activation.

IGFBP-3 Is Highly Expressed in Primary Esophageal Cancer Tissues. To determine IGFBP-3 expression in primary esophageal cancer tissues, real-time RT-PCR, Western blotting, and immunohistochemistry were performed. IGFBP-3 mRNA was overexpressed in 15 of 19 (78.9%) primary ESCC tissues, including 8 samples (36.8%) that showed a ≥10-fold increase compared with adjacent normal mucosa (Fig. 3A). IGFBP-3 overexpression was also observed in four of seven EAC tissues (57.1%; Fig. 3B). Western blotting confirmed IGFBP-3 overexpression in ESCC; 8 of 11 ESCC samples showed a ≥3-fold increase in IGFBP-3 expression compared with adjacent normal mucosa (Fig. 3C). The IGFBP-3 mRNA and protein levels tended to correlate with the EGFR protein level in the tumor tissues ($R^2 = 0.365; P = 0.003$). Concomitant up-regulation of EGFR and IGFBP-3 was observed in 7 of 11 (60%) samples of primary ESCC.

Finally, immunohistochemistry revealed that IGFBP-3 was intensely expressed in tumor tissues of 16 of 17 (94.1%) invasive ESCCs as well as 5 of 6 (83.3%) EACs (Fig. 4; data not shown). Whereas IGFBP-3 expression was increased in almost all layers of dysplastic squamous epithelia (Fig. 4B), it was confined to the basal and suprabasal cell layers of the normal esophageal squamous epithelium (Fig. 4A). IGFBP-3 was diffusely distributed in the cytoplasm of both ESCC and EAC (Fig. 4C–H). Interestingly, some foci of cancer cells had a very intense cytoplasmic staining for IGFBP-3 (Fig. 4H), which may represent localization of IGFBP-3 before secretion.
esophageal cancer cell lines expressed 30-fold or more IGFBP-3 mRNA than parental EPC2 cells at 3 PDs (Fig. 5A). It should be noted that presenescent EPC2 cells (42 PDs) expressed 10-fold more IGFBP-3 mRNA than EPC2 cells at early passages (3 PDs), consistent with observations by others about IGFBP-3 expression in senescent cells (10–12). IGFBP-3 mRNA was also increased in EPC1 (data not shown). When examining nonesophageal cancer cell lines, A431 and HaCaT cells expressed IGFBP-3 mRNA, whereas other cell lines, including HeLa, HepG2, PANC-1, and MCF7, did not (Fig. 5A; data not shown). Western blotting also demonstrated that 6 of 15 (40%) esophageal cancer cell lines expressed higher levels of IGFBP-3 than EPC2 cells (Fig. 5B).

It is possible that rapid secretion of IGFBP-3 into the cell culture medium after protein synthesis may explain the differences in RNA and protein overexpression. Therefore, we examined the CM for IGFBP-3 by Western blotting and ELISA. Western blotting showed that IGFBP-3 was detectable in concentrated CM (Fig. 5C), whereas other cell lines, including HeLa, HepG2, PANC-1, and MCF7, did not (Fig. 5A; data not shown). Western blotting also demonstrated that 6 of 15 (40%) esophageal cancer cell lines expressed higher levels of IGFBP-3 than EPC2 cells (Fig. 5B).

To determine whether IGFBP-3 produced by cell lines is functional, we performed Western ligand blotting. It is known that only full-length IGFBP-3, but not proteolytically cleaved versions, is capable of high-affinity binding to IGF-I (22, 23). Fig. 6A demonstrates that CM from A431 and TE2 cells contains proteins whose molecular masses are consistent with IGFBP-3 (40–44 kDa) and IGFBP-4 (24 kDa; ref. 21), which were documented by reprobing the ligand blot with specific antibodies (Fig. 6B and C), thus indicating that IGFBP-3 secreted by these cancer cell lines can bind to IGF-I.

EGFR Regulates IGFBP-3 Expression and Secretion. Because the ELISA data suggested that serum components may be responsible for IGFBP-3 secretion in most of the esophageal cancer cell lines and A431 cells with IGFBP-3 up-regulation (Fig. 5D), we next asked whether EGFR ligands in serum and corresponding EGFR activation are involved in regulation of IGFBP-3 expression in these cell lines. As shown in Fig. 7A, AG1478, an inhibitor of EGFR tyrosine kinase activity, partially inhibited IGFBP-3 secretion into CM of TE11 in a dose-dependent fashion but did not inhibit IGFBP-3 secretion into CM of TE2 and TE7 cells. Such an inhibitory effect of AG1478 on IGFBP-3 expression was further confirmed by ELISA and Western blotting in cell lysates as well as CM from A431 cells (data not shown), thereby indicating that serum in the medium can activate EGFR and lead to IGFBP-3 production in a subset of the cell lines. Furthermore, EGF induced IGFBP-3 expression in A431 cells under serum-deprived conditions (0.5% FCS), whereas AG1478 antago-

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**Fig. 3. IGFBP-3 is overexpressed in primary esophageal cancer tissues.** IGFBP-3 mRNA was determined by real-time RT-PCR in paired tissues of tumor and adjacent normal mucosa. Nineteen ESCC (A) and seven EAC (B) samples were analyzed. Mean ± SE (n = 3) in a representative experiment is shown. IGFBP-3 overexpression was based on ≥3-fold IGFBP-3 mRNA levels in tumor specimens compared with normal tissue specimens (3). EGFR and IGFBP-3 were determined in tissue lysates prepared from 11 paired clinical samples by Western blotting. T, tumor; N, adjacent normal tissue. Tubulin was used as a loading control.
nized this effect in a dose-dependent fashion (data not shown). EGF-mediated induction of IGFBP-3 was also observed in TE11 cells (Fig. 7B). Thus, these experiments suggest that EGFR activation leads to IGFBP-3 induction in A431 and TE11 cells. By contrast, EGF suppressed the basal level of IGFBP-3 expression in TE2 and TE7 cells in a dose-dependent manner when cells were serum starved overnight (Fig. 7D and F). Interestingly, the IGFBP-3 level was further increased in TE2 and TE7 cells when the cells were left serum starved for an additional 48 hours (Fig. 7D and F, Lanes 2). EGF also inhibited IGFBP-3 expression in TE12 cells in a dose-dependent fashion (data not shown). Moreover, EGF stimulation did not appear to affect the basal level of IGFBP-3 expression detectable in TE1 and TE8 cells based on Western blotting (data not shown). It should be noted that Western blotting (data not shown) confirmed the effects of EGF on EGFR tyrosine phosphorylation in these cell lines.

We further asked whether EGFR regulates the level of IGFBP-3 secreted in the CM. ELISA demonstrated that a concentration of 1 to 100 ng/mL EGF stimulates secretion of IGFBP-3 in TE11 cells (Fig. 7C) and A431 cells (data not shown). By contrast, 0.1 to 100 ng/mL EGF dose-dependently suppressed IGFBP-3 secretion in CM of TE2 and TE7 cells (Fig. 7E and G), whereas 0.1 to 10 pg/mL of EGF stimulated IGFBP-3 secretion (Fig. 7E and G), thus suggesting a biphasic effect of EGF on IGFBP-3 expression in these cell lines. Such changes in IGFBP-3 levels in CM on EGF stimulation appeared
to be consistent with our findings in cell lysates as demonstrated by Western blotting (Fig. 7B, D, and F; data not shown). These observations support the premise that EGFR activation regulates IGFBP-3 expression and secretion.

Inhibition of IGFBP-3 by Small Interfering RNA in TE11 Cells Augments Cell Proliferation. To gain insight into the biological role of IGFBP-3 in esophageal cancer cell lines, we stably transduced TE11 cells with siRNA directed against IGFBP-3, resulting...
Fig. 7. IGFBP-3 expression is regulated by EGFR activation. IGFBP-3 expression in cell lysates and CM was determined by ELISA (A, C, E, and G) and Western blotting (B, D, and F) after treatment with AG1478 (specific EGFR tyrosine kinase inhibitor), dimethyl sulfoxide as a control vehicle, or EGF at the indicated concentrations and for the indicated periods. A. TE2, TE7, and TE11 cells were incubated with or without AG1478 for 72 hours in the presence of 10% FCS, and CM was subjected to ELISA. B–G, TE11 (B and C), TE2 (D and E), and TE7 (F and G) cells were serum starved with serum-free medium for 16 hours and treated with or without EGF for 48 hours. *, P < 0.01.
in creation of IGFBP-3 siRNA-expressing TE11-S cells and empty vector-transduced TE11-N control cells. We confirmed by Western blotting that IGFBP-3 protein expression was suppressed by 70% in TE11-S cells compared with TE11-N cells (Fig. 8A). ELISA also revealed a 78% reduction in IGFBP-3 secretion in CM of TE11-S cells (Fig. 8B). When cell growth was assessed, TE11-S cells appeared to grow faster than control cells (Fig. 8C). This notion was documented by a \(^{[3]H}\)thymidine incorporation assay showing 14-fold more active DNA synthesis in TE11-S cells than in TE11-N cells (Fig. 8D). Moreover, DNA content analysis by flow cytometry revealed a decrease in G1 phase (10%) and an increase in S phase (10%) in TE11-S cells compared with TE11-N cells (data not shown). No sub-G\(_1\) fraction was observed in both cell lines when they were grown under standard tissue culture conditions or in a serum-starved condition for 48 hours (data not shown). The trypan blue dye exclusion test also detected no significant difference in cell viability rate between TE11-S and TE11-N cells. These observations indicate that IGFBP-3 may restrain TE11 cell proliferation without inducing apoptosis. However, we would emphasize that further investigation is needed.

**DISCUSSION**

An unbiased gene array-based screening allowed us to identify IGFBP-3 as a novel gene critically regulated in EPC2 cells that overexpress EGFR. This was further confirmed in independently transduced primary and hTERT-immortalized esophageal epithelial cells. We found that IGFBP-3 is up-regulated in primary and immortalized human esophageal cells transduced with EGFR and that IGFBP-3 is frequently overexpressed in esophageal cancer cell lines and primary esophageal cancer tissues. A subset of esophageal cancer cell lines expresses high levels of both IGFBP-3 and EGFR.

What is the basis for EGFR-mediated regulation of IGFBP-3? One possibility is that IGFBP-3 expression is subject to regulation by EGFR signaling. Additionally, EGFR overexpression in EPC2 cells induces a phenotypic change associated with growth inhibition, earlier senescence, or differentiation, culminating in up-regulation of IGFBP-3. Akerman *et al.* (24) observed that excessive EGFR signaling shortened the replicative life span of normal human skin keratinocytes. IGFBP-3 has been found to be up-regulated in senescent or quiescent human diploid fibroblasts (11), vascular endothelial cells (12), and prostate epithelial cells (14). Thus, IGFBP-3 induction in EPC2-EGFR and EPC2-hTERT-EGFR cells may be associated with growth inhibition in monolayer culture. By contrast, it is unlikely that IGFBP-3 up-regulation is a consequence of EPC2-EGFR cell differentiation because our gene array data did not show any changes for early and terminal keratinocyte differentiation markers, including cytokeratins K1/K10, K4/K13, involucrin, and profilaggrin (data not shown). In addition, EPC2-EGFR cells are capable of undergoing differentiation in organotypic culture (6). By contrast, IGFBP-3 up-regulation is associated with esophageal epithelial cell hyperproliferation induced by EGFR overexpression in both organotypic cell culture and transgenic mice (Fig. 2). Thus, regulation of IGFBP-3 by EGFR in a physiologic context may be different from that observed in monolayer cell culture.
IGFBP-3 is transcriptionally activated by p53 (25, 26) through several p53-binding cis-regulatory elements (25, 27). Given the role of p53 in cellular senescence (28), it is intriguing to speculate that p53 transcriptional activity is involved in IGFBP-3 induction in EPC2-EGFR cells. In fact, we have recently observed that p53<sup>R175H</sup>-inhibited IGFBP-3 mRNA up-regulation and prolonged the replicative life span of EPC2-EGFR cells (data not shown).

**Mechanisms for IGFBP-3 Overexpression in Esophageal Cancer**. Our finding of IGFBP-3 overexpression in esophageal cancer is the first such demonstration in this cancer type. Our data showed a significant correlation between mRNA levels and protein levels in cancer cell lines as well. It is noteworthy that EGFR and IGFBP-3 are localized in close proximity on human chromosome 7p12.3-p12.1 and 7p13-p12, respectively. Because EGFR gene amplification is noted in several esophageal cancer cell lines including TE3, TE5, and TE8 (29) and A431 cells, it is interesting to consider the possibility of co-amplification of the IGFBP-3 and EGFR loci. However, gene amplification seems to be the least likely mechanism for IGFBP-3 overexpression in the majority of cases because EGFR gene amplification is found in only 10% to 20% of primary esophageal cancer tissues that overexpress EGFR (5, 30). Hintz et al. (31) also found no chromosomal alterations such as recombination and gene amplification in primary tumors that overexpress IGFBP-3.

ELISA and Western blotting showed remarkable down-regulation of IGFBP-3 in most of the cell lines examined on serum deprivation (Fig. 5D), thus suggesting that serum factors play a role in regulation of IGFBP-3 expression in these cells. Serum deprivation could affect cell growth and thus production of IGFBP-3. An equivalent number of cells were seeded per plate to collect conditioned media for ELISA. However, differences in cell number on harvesting of conditioned media could affect the IGFBP-3 concentrations. Thus, we calibrated the ELISA data on conditioned media using total protein yield from simultaneously harvested cell lysates.

In this study, EGFR tyrosine kinase activity was involved in the regulation of IGFBP-3 expression in several esophageal cancer cell lines and A431 cells. A431 and TE11 cells exhibited partial dependency of IGFBP-3 expression on EGFR tyrosine kinase activity when they were grown in the presence of 10% FCS (Fig. 7A; data not shown). By contrast, suppression of EGFR kinase activity did not influence the IGFBP-3 level in TE2 and TE7 cells (Fig. 7A), suggesting that EGFR ligands in serum may not be the only factors contributing to IGFBP-3 induction. In addition, the IGFBP-3 level maximally induced in CM by EGF in TE11 and A431 cells appeared to be lower than that induced by 10% FCS (Figs. 5D and 7C; data not shown). In primary esophageal tumor tissues, IGFBP-3 expression level tended to correlate with EGFR level (Fig. 3C). However, three samples (ESCC9, ESCC13, and ESCC20) appeared to express an increased level of IGFBP-3 without EGFR overexpression (Fig. 3C), suggesting a separate mechanism accounting for the IGFBP-3 induction in these samples.

Numerous agents other than EGF have been shown to induce IGFBP-3 in vitro (9, 13). They include growth hormone, IGF-I, transforming growth factor β, tumor necrosis factor α, retinoic acid, and vitamin D. Thus, such peptide growth factors and hormones may contribute to IGFBP-3 induction in cell lines and primary tumor tissues. They may also affect cellular responses to EGFR ligands in modulating IGFBP-3 expression. For example, all-trans-retinoic acid induces IGFBP-3 in the presence of EGF at concentrations that suppress IGFBP-3 in cervical epithelial cell lines (32). Further complexity underlying EGFR-mediated regulation of IGFBP-3 was observed in that the effect of EGF stimulation on IGFBP-3 expression is different among the cell lines examined. EGF has been shown to inhibit IGFBP-3 mRNA and protein expression in primary and spon-

taneously immortalized human skin keratinocytes (33, 34). These observations are consistent with our observations in TE2 and TE7 cells when they were stimulated at nanogram levels of EGF (Fig. 7D–G). By contrast, picogram levels of EGF induced IGFBP-3 in these cell lines, albeit modestly (Fig. 7E and G). Furthermore, EGF potently induced IGFBP-3 expression and secretion in TE11 and A431 cells (Fig. 7B and C; data not shown). EGF-mediated induction of IGFBP-3 secretion has been demonstrated in fetal rat lung fibroblasts (35) and newborn rat astroblasts (36). Such a discrepancy in EGF response among cell lines may be accounted for by differences in EGFR level or activation. Additionally, we also found that higher cell density enhances EGF-mediated induction of IGFBP-3 expression in these esophageal cancer cell lines (data not shown).

Feldser et al. (37) recently showed that IGFBP-3 expression is prominently impaired in hypoxia-inducible factor (HIF)-1α<sup>−/−</sup> embryonic stem cells and that hypoxia induces IGFBP-3 mRNA in HIF-1α<sup>−/−</sup> embryonic stem cells. They also showed that hypoxia, as well as fibroblast growth factor and EGF, induces HIF-1α in 293 cells. Functional inactivation of the von Hippel-Lindau (VHL) gene, an essential component of E3 ubiquitin-ligase complex, is common in renal cancer and known to result in HIF-1α stabilization. However, VHL deficiency is not common in esophageal cancer (38–40). Treins et al. (41) showed that insulin activates HIF-1α through the phosphatidylinositol 3'-kinase/AKT pathway in retinal epithelial cells. Because EGFR activates AKT in esophageal cancer cell lines (19) and EPC2-EGFR cells (6), it is tempting to speculate that HIF-1α may play a role in IGFBP-3 induction in esophageal cancer cells as well as in primary and immortalized human esophageal cells transduced with EGFR.

Growth hormone, a potent inducer of IGFBP-3, activates Jak2 to induce tyrosine phosphorylation of EGFR, independent of its own ligands and intrinsic kinase activity, resulting in recruitment of Grb2 and activation of mitogen-activated protein kinases (42). Thus, it is also possible that growth hormone utilizes EGFR to induce IGFBP-3 in the absence of EGFR ligands. Interestingly, a prospective study on a cohort of 1,041 men with acromegaly revealed an increased incidence rate for esophageal cancer (43).

Our data also clearly suggest that p53 is unlikely to contribute to IGFBP-3 induction in cancer cell lines, contrary to primary human esophageal cells, because p53 mutation and/or loss of p53 functions have been documented in almost all esophageal cancer cell lines (44, 45).

**The Role of IGFBP-3 in Regulation of Esophageal Cell Proliferation and Apoptosis**. IGFBP-3 has been shown to both stimulate and inhibit cell proliferation under various experimental conditions through IGF-dependent and independent mechanisms (9, 13). However, IGFBP-3 plays a critical role in the induction of apoptosis of several cancer cells in a p53-dependent (46) and -independent (47, 48) fashion. In addition, many agents have been shown to inhibit breast cancer cell proliferation in vitro through induction and secretion of IGFBP-3 (48). They include tumor necrosis factor α, transforming growth factor β, retinoic acid, vitamin D, and antiestrogen compounds such as tamoxifen and IC1182,780. Recently, silibinin, a flavonoid antioxidant derived from milk thistle, has been shown to up-regulate IGFBP-3 to inhibit proliferation of prostate cancer cells (49). Interestingly, EGFR appeared to mediate the cytotoxic effect of silibinin on rat glioma cells (50).

Thus, the antiproliferative or proapoptotic activities of IGFBP-3 may provide a safeguard mechanism against carcinogenesis in certain tumor types. In fact, down-regulation of IGFBP-3 has been documented in several cancers, including prostate cancer (51) and hepatocellular carcinoma (52, 53). Consistent with these observations, IGFBP-3 was down-regulated in prostate epithelial cells immortalized...
by human papilloma virus E7 oncoprotein through a mechanism involving physical interaction between E7 and IGFBP-3, leading to polyubiquitination and proteolytic degradation of IGFBP-3 (14, 54), although another study (55) showed up-regulation of IGFBP-3 in cervical keratinocytes immortalized with human papillomavirus E6 and E7. Hanafusa et al. (56) showed epigenetic silencing of IGFBP-3 transcription through hypermethylation of the IGFBP-3 promoter as a mechanism for IGFBP-3 down-regulation in hepatocellular carcinoma.

Our findings of IGFBP-3 overexpression in esophageal cancer represent a sharp contrast to these previous observations. One interpretation is that IGFBP-3 may stimulate cell proliferation by enriching the IGF on the cell surface and thereby allowing it to activate the IGF receptor, following the proteolytic cleavage of IGFBP-3 mediated by enzymes such as matrix metalloproteases (9, 57). Another possibility is that IGFBP-3 was induced in esophageal cancer cells as a result of exposure to various growth factors, hormones, and cellular stress such as hypoxia and therapeutic agents in tumor tissues. However, a subset of these cancer cells acquires genetic alterations to negate the growth-inhibitory effect of IGFBP-3. Consistent with such a notion is the finding that growth inhibition on excessive EGFR activation observed in A431 cells is not found in the majority of esophageal cancer cell lines (58). Another possibility is that IGFBP-3 inhibits tumor cell proliferation as a negative feedback mechanism. EGFR treatment of human papilloma virus type 16-immortalized ectocervical epithelial cell lines results in decreased IGFBP-3 expression and concomitant enhancement of cell proliferation (32, 59). Thus, it is possible that EGF-induced down-regulation of IGFBP-3 in TE2 and TE7 cells may facilitate the mitogenic effect of EGF in these cell lines.

It is noteworthy that IGFBP-3 up-regulation was observed in esophageal squamous dysplasia by immunohistochemistry. It should be emphasized that IGFBP-3 has been demonstrated to enhance EGFR phosphorylation, activation of p44/42 and p38 mitogen-activated protein kinases, and cell proliferation in MCF10A breast epithelial cells stimulated with 0.1 to 1 ng/mL EGF (60). Because EGFR overexpression is one of the earliest genetic changes found in esophageal squamous dysplasia by immunohistochemistry. It should be emphasized that IGFBP-3 inhibits tumor cell proliferation as a negative feedback mechanism. EGF treatment of human papilloma virus type 16-immortalized ectocervical epithelial cell lines results in decreased IGFBP-3 expression and concomitant enhancement of cell proliferation (32, 59). Thus, it is possible that EGF-induced down-regulation of IGFBP-3 in TE2 and TE7 cells may facilitate the mitogenic effect of EGF in these cell lines.

In aggregate, we have identified IGFBP-3 as an aberrantly regulated gene through the EGFR signaling pathway. IGFBP-3 overexpression in esophageal cancer implies a role in esophageal carcinogenesis through IGF-dependent or independent actions. Additional studies are required to elucidate how IGFBP-3 affects cellular functions, such as proliferation, differentiation, and migration, and the interplay between EGFR and IGFBP-3.

ACKNOWLEDGMENTS

We thank Dr. Yasuhiro Shirakawa (Okayama University, Okayama, Japan) for clinical samples. We thank Dr. Don Baldwin (Microarray facility at the University of Pennsylvania, School of Medicine) for gene array experiments, Dr. Gary Swain (Morphology Core facility) for immunohistochemistry, Dr. Sue A. Keilbaugh and Weinian He (Molecular Biology Core facility) for technical assistance in RNA isolation and real-time RT-PCR, Caitlin Smith and Michael K. Mashiba for technical assistance in ELISA and Western blotting, and Drs. Therese B. Deramond, Cameron N. Johnstone, and Ben Rhodes (Rustgi laboratory) for helpful discussions at the University of Pennsylvania, Philadelphia, PA. Finally, we are grateful to Drs. Meenhard M. Herlyn (Wistar Institute, Philadelphia, PA) and Anil K. Rustgi (Gastroenterology Division, University of Pennsylvania) for their support and advice and for critical review of the manuscript by Dr. Rustgi.

REFERENCES


Epidermal Growth Factor Receptor Regulates Aberrant Expression of Insulin-Like Growth Factor-Binding Protein 3

Munenori Takaoka, Hideki Harada, Claudia D. Andl, et al.

Cancer Res 2004;64:7711-7723.

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